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Autocrine receptors for endothelins in the primary culture of endothelial cells of human umbilical vein

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Human umbilical vein endothelial cells (HUVECs) in primary culture produced and secreted endothelin 1 (ET-1) actively. Specific binding of [^{125}I]ET-1 to these cells was not detectable because of the saturation of ET receptors with endogenously produced ET-1. However, addition of phosphoramidon, an inhibitor of ET-converting enzyme, to the medium reduced the production of ET-1 and thus the receptors on HUVECs were made available for exogenously added [^{125}I]ET-1. Binding studies using phosphoramidon-treated HUVECs indicated the existence of a non-isopeptide-selective type (ET_B) of ET receptor with a K_d of 17 pM. This receptor is thought to be involved in ET-induced vasodilation in an autocrine manner in vivo.

Endothelin; Endothelial cell; Receptor; Vasodilation

1. INTRODUCTION

Endothelin, now known as ET-1, was first purified from the culture medium of porcine endothelial cells and is the most potent and longest acting constrictor of vascular and non-vascular smooth muscle cells [1]. Subsequently, ET-1 has been shown to be a member of a family consisting of three isopeptides (ET-1, -2, and -3), which exhibit distinct pharmacological actions [2]. Their actions are mediated by two receptor subtypes, i.e. ET-1- and ET-2-selective ET_A and non-isopeptide-selective ET_B [3–6].

ETs stimulate endothelial cells (ECs) to secrete two well-characterized vasodilator substances, prostacyclin [7,8] and endothelium-derived relaxing factor [8,9]. ET-1 and ET-2 are much more potent than ET-3 in their vasoconstrictive effects, whereas the three ETs are almost equipotent in their vasodilatory effects. It is, therefore, likely that ET receptor on ECs are of the ET_B subtype. However, the nature of ET receptors on ECs still remains controversial. Emori et al. [10] reported that the ET receptors in cultured bovine aortic ECs are of ET_C (ET-3-specific) subtype (but not ET_B subtype) based on the observation that [^{125}I]ET-3 binding is not displaced by unlabeled ET-1. Takayanagi et al. [11], on the other hand, suggested the presence of ET_B receptor on ETs from the finding that specific binding sites for ^{125}I-labeled [Glu^9] sarafotoxin S6b, a ligand relatively specific for ET_B receptor, are detected in membranes of endothelium-containing rat thoracic aorta, but become almost undetectable after removal of the endothelium. Furthermore, Ogawa et al. [6] detected significant expression of ET_B receptor mRNA in human umbilical vein endothelial cells (HUVECs).

In this paper, we show that ET receptors on HUVECs in primary culture are saturated with endogenous ET-1 and unable to bind exogenously added [^{125}I]ET-1. When the production of endogenous ET-1 is inhibited by treating the cells with phosphoramidon, an inhibitor of ET-converting enzyme [12], the receptors become detectable by binding assay with [^{125}I]ETs. Using this strategy we have been able to demonstrate that the receptors on HUVECs are of the ET_B (non-isopeptide-selective) subtype.

Abbreviations: EC, endothelial cell; ET, endothelin; HBSS, HEPES-buffered salt solution; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; HUVECs, human umbilical vein endothelial cells; RIA, Radioimmunoassay.

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2. MATERIALS AND METHODS

2.1. *Cell culture*

HUVECs (2nd passage) were purchased from Kurabo (Osaka, Japan) and cultured on 6-well plates at 37°C in a CO_2 incubator (5% CO_2, 95% air) as previously reported [13]. After reaching confluency, various concentrations of phosphoramidon (Peptide Institute Inc., Osaka, Japan) were added with fresh media every 24 h for a further 2 days. Experiments were performed on the 3rd day after reaching confluency. All cells used in these experiments were subcultured 3 times.

2.2. *Binding assay*

Phosphoramidon-treated and untreated HUVECs were washed with 20 mM HEPES (pH 7.4), containing 140 mM NaCl, 4 mM KCl, 1 mM K_2HPO_4, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM D-glucose and 0.05% bovine serum albumin (HBSS). The cells were incubated in HBSS with 50 pM [^{125}I]ET-1 or [^{125}I]ET-3 (~74 TBq/mmol, Amersham)

to obtain total binding. Non-specific binding was determined in the presence of 100 nM unlabeled ET-1 or ET-3. Specific binding was defined as total binding minus non-specific binding. After incubation at 37°C for 1 h, the reaction was stopped by aspiration of the reaction buffer. The cells were washed twice with ice-cold HBSS and solubilized in 1N NaOH. The cell-associated radioactivity was measured in a Wallac-1470 Wizard autogamma counter (Pharmacia).

2.3. *Determination of ET-1 and big ET-1 in the culture medium*

ET-1 and big ET-1 were purified from the culture medium of HUVECs by adsorption onto an Amprep ethyl C2 minicolumn (Amersham), followed by reversed-phase high-performance liquid chromatography [12]. Fractions corresponding to ET-1 and big ET-1 were collected, evaporated and used for radioimmunoassay (RIA) with an [^{125}I]ET RIA kit (Amersham).

ET-1 was also determined by a receptor binding assay using porcine lung membranes. Porcine lung was homogenized with a Kinematica Polytron homogenizer (Lucern, Switzerland) three times at top speed for each 30 s in 9 vols of ice-cold 0.25 M sucrose solution containing 20 mM Tris-HCl (pH 7.4). After centrifugation of the homogenate at 1,000 × *g* for 10 min at 4°C, the supernatant was centrifuged again at 20,000 × *g* for 20 min. The resulting pellet was washed three times as described above and used as a membrane preparation. The membrane (10 μg protein) was incubated with 10 pM [^{125}I]ET-3 in the presence of serial dilutions of the conditioned medium (<200 μl) in a total volume of 1 ml of HBSS. After incubation at 37°C for 1 h, the membrane-associated radioactivity was recovered by centrifugation and counted. The calibration curve was obtained with known amounts of authentic ET-1.

2.4. *Identification of ECs*

The ECs were identified by immunofluorescent staining with mouse antibody against vector-VIII (Synbiotics Corp., USA), an endothelial marker protein, and fluorescein isothiocyanate-labeled anti-mouse IgG antibody (Kirkegaard & Perry Labs. Inc., USA). The viability of ECs was examined by a dye exclusion test with 0.01% Trypan blue.

2.5. *Light microscopic radio-autography*

HUVECs cultured on slide-glass wells were incubated at 37°C for 1 h with 50 pM [^{125}I]ET-1 or [^{125}I]ET-3 in the presence of absence or 100 nM unlabeled ET-1 or ET-3 as described above. Then, the cells were rinsed twice in HBSS and fixed with 0.75% glutaraldehyde in 0.1 M sodium phosphate (pH 7.5) for 10 min. After dehydration in 50% ethanol for 15 min, slides were air-dried, dripped into Konica NR-M2 emulsion and stored in the dark. After exposure for 20–35 days, the slides were developed with Konidol X for 6 min at 25°C and observed under a microscope.

3. RESULTS AND DISCUSSION

As reported previously [12], HUVECs produced and secreted ET-1 activity when cultured in the absence of phosphoramidon; the ET-1 content in the 24-h conditioned medium was determined by RIA to be 1,000 fmol/10^6 cells. Under these conditions, practically no big ET-1 was detected (<10 fmol/10^6 cells) and specific binding of 50 pM [^{125}I]ET-1 to the cells was negligible (<0.1 fmol/10^6 cells). When the cells were cultured in the presence of 12 mM phosphoramidon, which did not affect the cell number, viability of morphology of HUVECs, the content of ET-1 in the 24-h conditioned medium decreased to 450 fmol/10^6 cells whereas that of big ET-1 increased to 560 fmol/10^6 cells. The sum of ET-1 and big ET-1 contents was almost constant at all the phosphoramidon concentrations tested (Fig. 1), and was approximately the same as the ET-1 content observed when the cells were cultured in the absence of the inhibitor. These results indicated that phosphoramidon specifically inhibited the conversion of big ET-1 to ET-1 catalyzed by ET-converting enzyme, as reported previously [12,14]. However, the concentration of phosphoramidon which inverted the ratio of ET-1/big ET-1, determined for HUVECs (4 mM) was much higher than that reported for bovine aortic ECs (3–10 μM) [14].

The phosphoramidon-induced decrease in ET-1 accumulation was accompanied by the appearance of specific binding sites for 50 pM [^{125}I]ET-1 on HUVECs (3.3 fmol/10^6 cells at 12 mM phosphoramidon). The extent of this appearance was dependent on the phosphoramidon concentration used (Fig. 1). It was thus evident that in the absence of the inhibitor the binding sites for ET-1 were saturated with endogeneous ET-1 actively produced by HUVECs themselves and, therefore, no further binding of [^{125}I]ET-1 could occur.

The decrease in ET-1 content after phosphoramidon treatment was also confirmed by a receptor-binding assay using porcine lung membranes (Fig. 2); the media after 24-h culture of HUVECs in the presence or absence of 4 mM phosphoramidon inhibited the [^{125}I]ET-3 binding dose-dependently; the inhibition was more pronounced with the medium after culture without phosphoramidon. From the slopes of inhibition curves, the ET-1 content in the medium was calculated to be 500 fmol/10^6 cells without phosphoramidon and 200 fmol/10^6 cells with 4 mM phosphoramidon. The ET-1 contents determined by this method were about 45% of those determined by RIA. The reason for this discrepancy is unclear, but the possibility exists that RIA overestimated ET-1 because of the presence of degradation products of ET-1 [15], which can be recognized by the antibodies but not by the ET receptor.

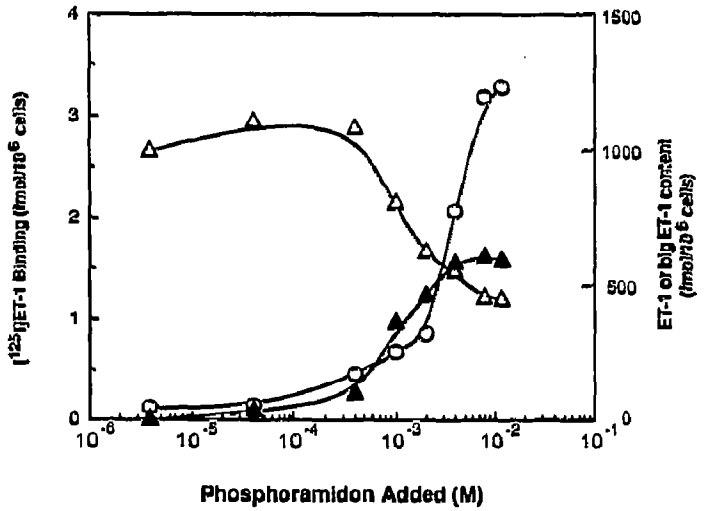

Fig. 1. Effects of phosphoramidon, at various concentrations, on the contents of ET-1 (△) and big ET-1 (▲) in the cultured medium and the specific binding of 50 pM [^{125}I]ET-1 to HUVECs (○). Each point is the mean of triplicate determinations.

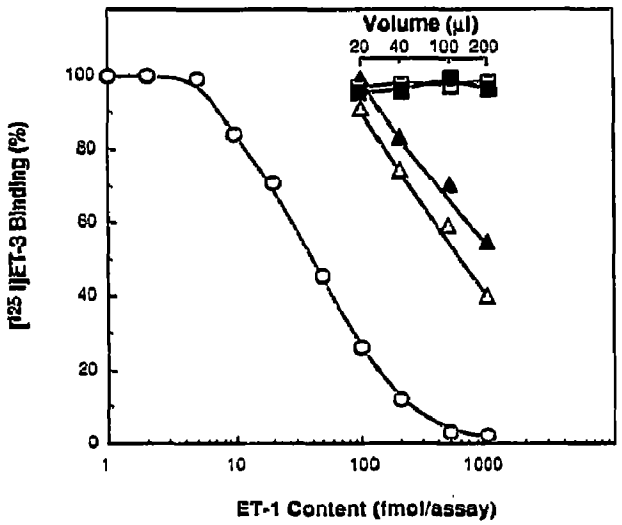

Fig. 2. Receptor binding assay for ET-1-like material in the culture medium of HUVECs. Shown is the displacement of 10 pM [^{125}I]ET-3 binding by increasing concentrations of ET-1 in porcine lung membranes (○). The inset shows the displacement of ET-3 binding in porcine lung membranes by the indicated volumes of unconditioned (□, ■) or HUVEC-conditioned culture medium (△, ▲). Data were obtained in the absence (□, △) or presence (■, ▲) of 4 mM phosphoramidon. Each point is the mean of triplicate determinations.

Another possibility is that ET-1 in the medium was partly trapped by unknown substances and, therefore, could not bind to the receptor.

The cell number of HUVECs in the culture was unchanged by the addition of 4 mM phosphoramidon. More than 90% of cells were immunoreactive with anti-factor VIII antibodies both in the presence and absence of phosphoramidon (data not shown). Autoradiographs of HUVECs cultured with 4 mM phosphoramidon, when incubated with radiolabeled ET-1 or ET-3, showed densely associated specific labeling in more than 90% of the cells (Fig. 3A). The radioactive deposits in the phosphoramidon-treated cells decreased significantly by co-incubation with 100 mM unlabeled ET-1 or ET-3 (data not shown). However, the cells cultured in the absence of phosphoramidon exhibited no specific labeling even after incubation with 50 pM [^{125}I]ET-1 or [^{125}I]ET-3 (Fig. 3B). These results indicated that the appearance of binding sites for radiolabeled ET-1 or ET-3 after phosphoramidon treatment was not due to selective growth of a minor population of non-endothelial cells.

We then attempted to characterize this binding. The Scatchard plot of [^{125}I]ET-1 binding to HUVECs treated with 4 mM phosphoramidon (Fig. 4A) revealed a single component of high affinity binding sites for ET-1 with a dissociation constant (K_d) of 17 pM and a maximum binding capacity (B_{max}) of 4.8 fmol/10^6 cells. With [^{125}I]ET-3 as ligand, almost the same K_d and B_{max} values were obtained as those for [^{125}I]ET-1. In competitive binding assays with 50 pM [^{125}I]ET-1, unlabeled ET-1 and ET-3 showed almost identical displacement curves with an IC_{50} of 100 pM (Fig. 4B), indicating that the non-isopeptide-selective type of ET receptor (ET_B)

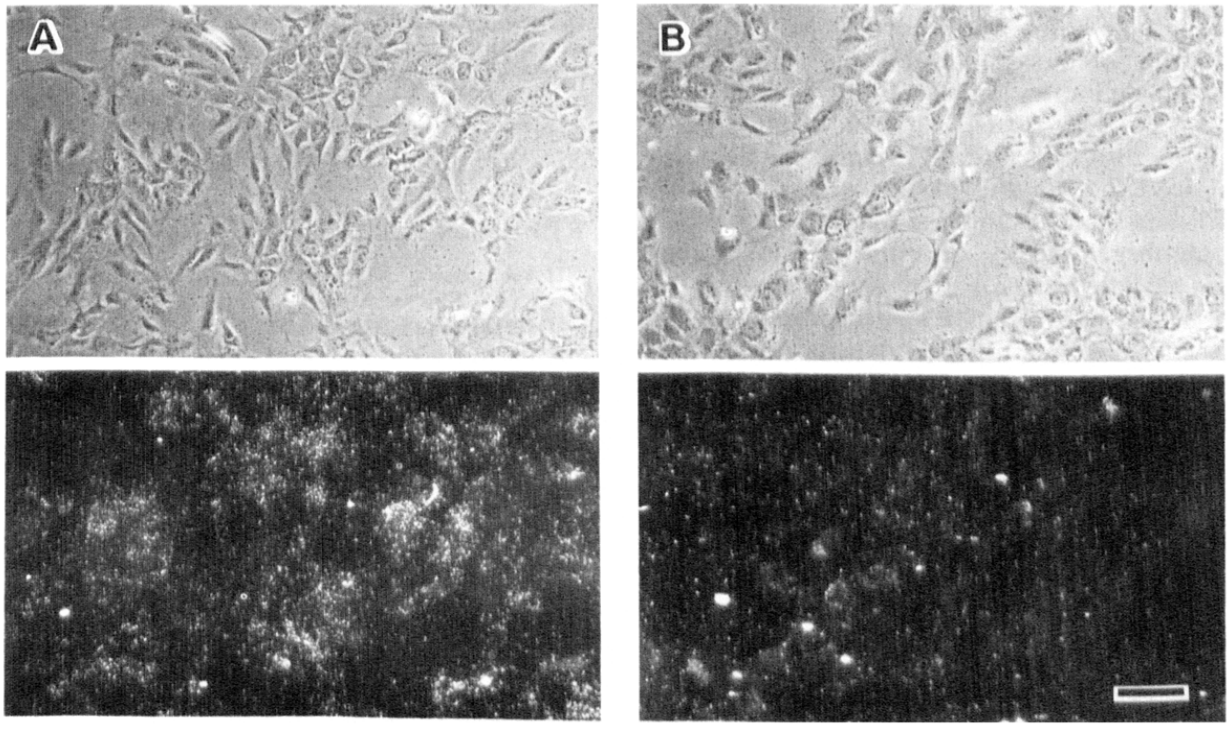

Fig. 3. Autoradiograms of HUVECs cultured in the presence (A) or absence (B) of 4 mM phosphoramidon after incubation with 50 pM [^{125}I]ET-1. This experiment was performed with HUVECs under a semi-confluent condition. Essentially identical autoradiograms were obtained with [^{125}I]ET-3. (Upper panels) light field observation; (lower panels) dark field observation. Bar, 100 μm.

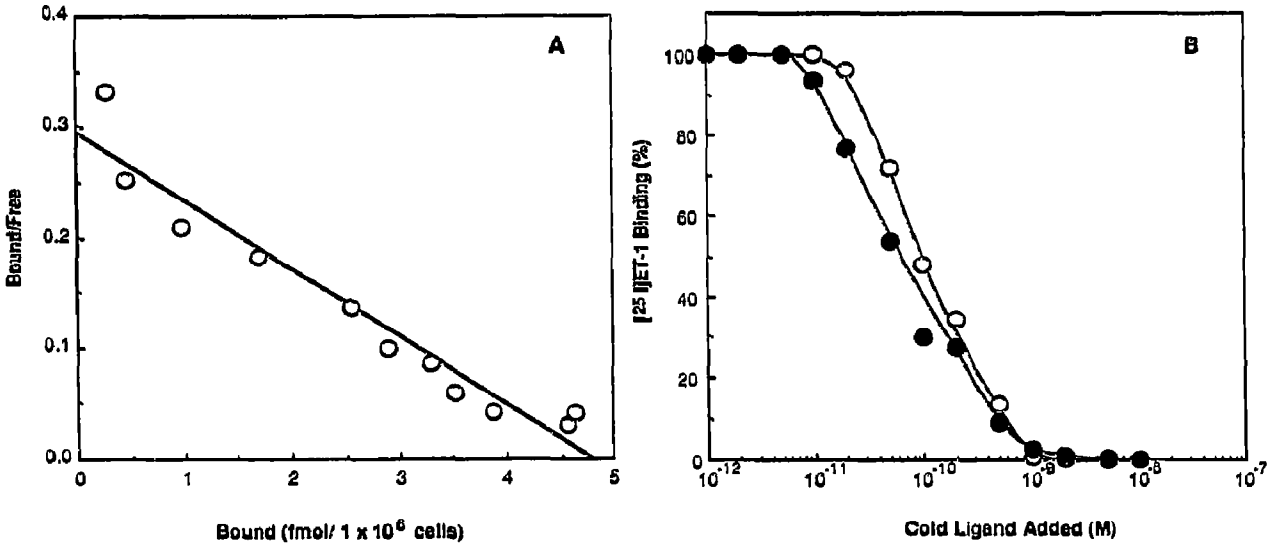

Fig. 4. (A) Scatchard analysis of the specific binding of [^{125}I]ET-1 to HUVECs cultured in the presence of 4 mM phosphoramidon. (B) Competitive binding of 50 pM [^{125}I]ET-1 to HUVECs cultured in the presence of 4 mM phorphoramidon by unlabeled ET-1 (○) and ET-3 (●). Results are expressed as percentages of the specific binding. Values are the means of triplicate determinations.

is responsible for the binding of ETs to HUVECs. Big ET-1, up to 10 μM, did not significantly displace the [^{125}I]ET-1 binding (data not shown).

The K_d value for the endothelial ET_B receptor (17 pM) was lower than that for the ET_A receptor (58 pM) determined in A-10 cells derived from rat aortic smooth muscle. This is in good agreement with pharmacological observations that vasodilation can be induced by smaller amounts of ETs as compared with vasoconstriction [9]. The specific binding sites for 50 pM [^{125}I]ET-1 (3.3 fmol/10^6 cells) obtained when the cells were treated with 12 mM phosphoramidon was about 1.5-fold that determined for the cells treated with 4 mM phosphoramidon (2.1 fmol/10^6 cells) (Fig. 1). Since the ET_B receptor on HUVECs was still partly occupied by endogenous ET-1 even at the inhibitor concentration of 12 mM, the number of ET_B receptor expressed on HUVECs is calculated to be >5,000 sites/cell, which is comparable to the number of ET_A receptor on A-10 cells (21,000 sites/cell) (unpublished data).

It can thus be concluded that HUVECs actively produce and secrete ET-1 and simultaneously express ET_B receptor as the major population, indicating that the receptor is an autocrine one. Cultured ECs from rat brain microvessels have been reported to possess ET receptors [16,17]. However, they are not involved in autocrine functions, because these ECs are unable to produce ETs.

In preliminary experiments we found that 100 nM ET-1 or ET-3 stimulated the release of prostacyclin from phosphoramidon-treated HUVECs but not from the untreated cells, suggesting that the endothelial ET_B receptor is involved in vasodilation as postulated previously [7,9]. Thus, it is likely that ET-1 secreted from ECs plays an important role as a local, autocrine vasomodulator by binding to ET_B receptor on ECs themselves. In this function ET-1 releases prostacyclin and endothelium-derived relaxing factor (NO) from ECs and thus attenuates the contraction mediated by smooth muscle ET_A receptor. Such a natural, negative-feedback mechanism would balance the strong, sustained contractions invokved by ET-1.

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